THE EFFECT OF SOME ANTIBIOTICS ON PROTEIN SYNTHESIS ACTIVITY OF IN VITRO RECONSTITUTED ROUGH MEMBRANE FROM RAT LIVER

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Received 14 April 1975

1. Introduction

Polyribosomes exist in rat liver cells in two different forms, as free polyribosomes and as polyribosomes bound to the endoplasmic reticulum [1]. In order to learn about the ribosome–membrane interaction, rough membranes have been stripped from their ribosomes by different methods and experiments have been carried out to reconstitute the rough membrane from these stripped membranes and polyribosomes [2,3]. Ribosomes do reattach to the stripped rough membrane but the major question is whether the reconstituted rough membrane is identical to the original rough membrane before it was stripped.

There are several reports about some differences between the amino acid incorporation activity of free polyribosomes compared to that of the rough membrane ribosomes, differences such as optimal Mg^{++} concentration [4] and sensitivity towards protein synthesis inhibitors [5]. In this communication we compare the effect of some inhibitors on protein synthesis promoted by rough to that of in vitro reconstituted rough membrane.

2. Materials and methods

Preparation of rat liver fractions: Free polyribosomes (FP), rough (RM) and smooth (SM) endoplasmic membranes were prepared as described by Czosnek and Hochberg [6]. For reconstitution purpose, rough membrane was stripped using the KCl-puromycin method (RMst) and free polyribosomes were bound to this stripped rough membrane according to Borgese et al. (RMrec) [3]. The different membrane fractions were pelleted and suspended in 5 ml 2 M sucrose TKM (Tris–HCl pH=7.6, 50 mM; KCl. 25 mM; $MgCl_2$, 10 mM); the suspension was underlaid below 24 ml of a 0.9 –1.9 M sucrose TKM continuous gradient. The gradients were spun for 14 hr at 24 000 rev/min in a SW 25.1 Beckman rotor. After the isopycnic flotation, the membrane band was aspired, pelleted at 78 000 *g* for 30 min and suspended in 1 M sucrose TKM. The purity of the membrane fractions was checked by measuring the RNA: protein ratio, buoyant density, amino acid incorporation capacity (see results), and also by electron microscopy using both transmission and freeze etching techniques [7].

The incorporation mixture (37°C) contained, in a final volume of 110 μl, the following: Tris–HCl, pH=7.4, 60 mM; KCl, 50 mM; $MgCl_2$, 10 mM; ATP, 0.5 mM; GTP, 0.5 mM; PEP, 5 mM; PEP kinase, 1 μg protein; tRNA (rat liver), 10 μg; poly U when added, 20 μg; non-radioactive amino acid mixture (minus leucine or minus phenylalanine), 5×10^{-2} mM each; and aliquots of the corresponding enzyme fractions (necessary for optimal incorporation); [^{3}H]leucine, 2 μCi or [^{3}H]phenylalanine, 1 μCi; free polyribosomes, 1.0 A_{260} nm unit or membrane fraction, 87 μg protein. At the indicated time intervals, samples were withdrawn and the radioactivity of the hot 5% TCA insoluble material was determined according to Bollum et al. [8].

Protein was determined according to Lowry et al. [9] and RNA was measured according to Bloemendal et al. [10].

3. Results and discussion

Fig.1. shows the isopycnic flotation pattern of the rough and the smooth membrane fractions isolated from rat liver, and of the stripped and the reconstituted rough membrane; RNA: protein ratios of the different fractions are indicated in the figure. From fig.1. it can be seen that the buoyant density and the RNA: protein ratio of the stripped rough membrane are equal to that of the smooth membrane; while after the incubation of the stripped rough membrane with free polyribosomes, a membrane fraction was obtained with a buoyant density and a RNA: protein ratio equal to that of the original membrane. Table 1 shows the amino acid incorporation activity of the different fractions. The smooth membrane and the stripped rough membrane have only a small amino acid incorporation activity compared to that of the other fractions. These incorporation activities can be correlated with the electron microscopy pictures, where only a few ribosomal particles can be seen in the smooth membrane or in the stripped rough membrane fraction. The reconstituted rough membrane has an amino acid incorporation activity similar to that of the rough membrane.

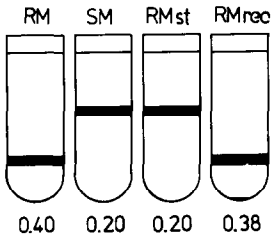

Fig.1. Isopycnic flotation of rough (RM), smooth (SM), stripped rough (RMst) and reconstituted rough membrane (RMrec) on continuous sucrose gradient. RM, SM, RMst: each membrane (20 mg protein) was pelleted, suspended in 2 M sucrose TKM and centrifuged as described in Material and methods; RMrec: stripped rough membrane (20 mg protein) was incubated for 30 min with 400 A_{260} units of free polyribosomes at 0°C, whereafter the mixture was pelleted and analyzed in the same way. The RNA: protein ratio of each fraction is given.

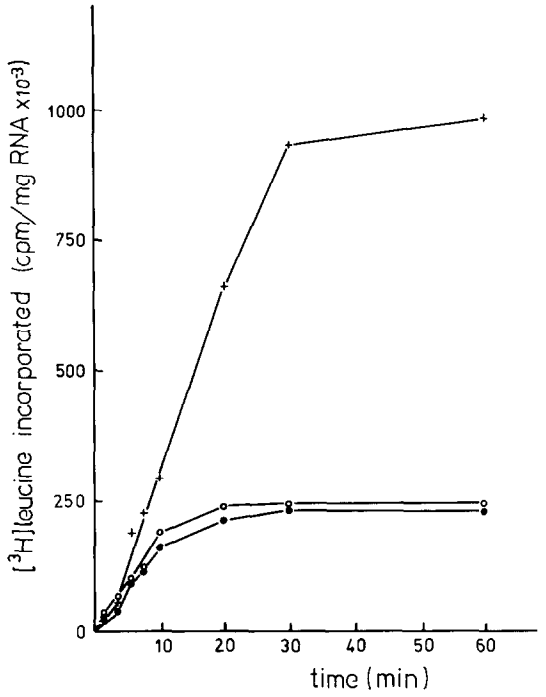

Fig.2. The kinetics of [^{3}H] leucine incorporation catalysed by free polyribosomes, rough membrane and reconstituted rough membrane.
(+—+) free polyribosomes, (○—○) rough membrane, (●—●) reconstituted rough membrane.

Fig.2 shows the kinetics of the amino acid incorporation activity of the polyribosomes, the rough and the

Table 1
Incorporation of [^{3}H]phenylalanine and [^{3}H]leucine into 5% hot TCA insoluble material, by different fractions

Incubation conditions	FP	RM	RMst	RMrec	SM
Phenylalanine - poly U	62 904	30 518	3816	18 562	3612
Phenylalanine + poly U	106 583	196 070	4000	145 068	8929
Leucine	138 927	83 872	5280	94 548	14 012

For incorporation assay see: Materials and methods. Results are expressed as cpm/mg protein.

Table 2
Effect of protein synthesis inhibitors on amino acid incorporation activity of free polyribosomes rough membrane and reconstituted rough membrane

Fraction		FP				RM				RMrec			
Incubation time (min)		2	5	10	30	2	5	10	30	2	5	10	30
Inhibitor concentration	(mM)												
	0.2	136	122	91	60	17	27	38	49	48	41	35	60
Aurin-	0.3	105	112	68	62	11	13	21	36	29	31	26	41
tricarboxylic	0.4	70	73	42	30	6	8	13	20	17	22	19	33
acid	0.5	75	50	27	17	2	4	7	9	12	10	9	17
	1.0	58	70	60	70	29	41	69	92	27	48	46	82
Cyclo-	2.0	39	42	40	43	25	35	44	67	30	33	30	67
heximide	3.0	28	36	36	37	21	22	35	58	30	30	26	62
	5.0	25	32	24	27	15	20	33	59	20	46	19	43
	20.0	68	87	95	75	30	56	90	99	51	56	69	100
Sodium	30.0	34	63	58	67	8	26	55	76	19	27	34	79
fluoride	40.0	14	32	30	33	7	16	40	68	12	15	20	52
	50.0	6	19	22	28	5	13	29	42	9	12	80	38
	1.0	45	68	72	72	25	53	82	100	40	58	66	98
Fusidic	2.0	30	47	41	29	15	33	57	84	29	39	54	100
acid	3.0	16	22	14	8	3	20	26	32	14	19	24	47
	4.0	0	3	0	0	2	0	0	0	0	0	0	3

The results are expressed as the percent of the control values (incorporation of [^{3}H]leucine without inhibitor)

reconstituted rough membrane fractions. The ratio of incorporation by the free polyribosomes remains constant for 20–30 min, but the rate of incorporation of the two membrane fractions drops sharply after 10 min and practically comes to a standstill after 20 min.

Only ribosomes bound to the membrane show this difference in the incorporation kinetics. The presence of rough membrane, per se, in an incorporation system containing an excess of free polyribosomes, does not change the rate of incorporation of these free polyribosomes (results not shown).

Table 2 shows the effect of four protein synthesis inhibitors on the amino acid incorporation capacity of the free polyribosomes, the rough and the reconstituted rough membrane. The results obtained with aurin tricarboxylic acid are very striking; at a concentration of 0.3 mM, or less, the initial rate of amino acid incorporation catalyzed by the free polyribosomes is not diminished, while that of the rough membrane fraction was diminished to an extent of 80–90%, and that of the reconstituted rough membrane to an extent of 70%. Cycloheximide decreases the initial rate of amino acid incorporation of the three fractions to nearly the same extent but, at a concentration of 1 mM the membrane fractions are somewhat more inhibited. The amino acid incorporation system of the membrane fractions are also more sensitive towards sodium fluoride. Fusidic acid showed only a slight discriminatory effect and inhibits the rough membrane system somewhat more than the polyribosomal incorporation system. In all the cases the discriminatory effects are more prominent if the incorporation assays are done during the linear period of the incorporation reaction. While the incorporation of amino acids by the membrane fractions reaches a maximum value after 10 min of incubation, in the presence of fusidic acid or sodium fluoride the incorporation continues after 10 min and reaches a maximum after 30 min. From the results described here it

is clear that the two membrane fractions, the rough membrane and the reconstituted rough membrane, behave very similarly in respect to the response of their amino acid incorporation activity towards the protein synthesis inhibitors. They are more sensitive towards these inhibitors than the free polyribosomes. It can also be seen that the amino acid incorporation activity of the reconstituted rough membrane is somewhat less inhibited by the inhibitors tested than the incorporation catalyzed by the rough membrane fraction. Therefore, according to these results, one is inclined to conclude that in the reconstituted rough membrane, the ribosome membrane interaction seem to be very similar, but may be not identical to the ribosome-membrane interaction in the rough membrane.

Acknowledgements

We wish to thank Mrs Tamar Schneider for her excellent technical assistance.

This work was supported by grant no. 173 from the United States–Israel Binational Science Foundation.

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